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(54) Title: METHOD FOR THE DETECTION OF THE ANTIBIOTIC RESISTANCE SPECTRUM OF MYCOBACTERIUM SPECIES

(57) Abstract

Method for the detection of the antibiotic resistance spectrum of *Mycobacterium* species present in a sample, possibly coupled to the identification of the *Micobacterium* species involved, comprising the steps of: (i) if need be releasing, isolating or concentrating the polynucleic acids present in the sample; (ii) if need be amplifying the relevant part of the antibiotic resistance genes present in said sample with at least one suitable primer pair; (iii) hybridizing the polynucleic acids of step (i) or (ii) with at least one of the rpoB gene probes, as specified in table 2, under the appropriate hybridization and wash conditions; (iv) detecting the hybrids formed in step (iii); (v) inferring the *Mycobacterium* antibiotic resistance spectrum, and possibly the *Mycobacterium* species involved from the differential hybridization signal(s) obtained in step (iv).

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METHOD FOR THE DETECTION OF THE ANTIBIOTIC RESISTANCE SPECTRUM OF MYCOBACTERIUM SPECIES

The present invention relates to the field of drug-resistant mycobacteria.

The present invention relates to probes. primers. methods and kits comprising the same for the detection of mycobacterial nucleic acids in biological samples.

Identification of most clinically relevant Mycobacterium species. in particular of Mycobacterium tuberculosis is tedious and time consuming due to culture-procedures which can take up to 6 weeks. Rapid diagnosis of Mycobacterium infection is very important since the disease might be life-threatening and highly contagious. Only recently some methods - all making use of one or another amplification process - have been developed to detect and identify Mycobacterium species without the need for culture (Claridge et al., 1993). Most of these methods are still in evaluation and their benefit in routine applications remains questionable. Moreover. these methods do not solve the problem of Mycobacterium drug-resistance detection which still relies on culture.

Since the frequency of multidrug resistance in tuberculosis is steadily increasing (Culliton, 1992). it is clear now that early diagnosis of M. tuberculosis and the rapid recognition of resistance to the major tuberculostatics are essential for therapy and an optimal control of the resurgent epidemic.

The antibiotics used for treatment of M. tuberculosis infections are mainly isoniazid and rifampicin either administered separately or as a combination of both. Occasionally, pyrazinamide. ethambutol and streptomycin are used: other classes of antibiotics like (fluoro)quinolones may become the preferred tuberculostatics in the future.

Since most multidrug resistant mycobacteria also lost susceptibility to rifampicin. rifampicin-resistance is considered to be a potential marker for multidrug resistant tuberculosis. For this reason. the detection of resistance to rifampicin might be of particular relevance.

For the majority of the M. tuberculosis strains examined so far. the mechanism responsible for resistance to rifampicin (and analogues like rifabutin) has been elucidated. Rifampicin (and analogues) block the RNA polymerase by interacting with the ß-subunit of this enzyme. Telenti et al. (1993a) found that mutations in a limited region of the ß-subunit of the RNA polymerase of M. tuberculosis give rise to insensitivity of the RNA polymerase

for rifampicin action. This region is limited to a stretch of 23 codons in the rpoB gene. The authors describe 17 amino acid changes provoking resistance to rifampicin (Telenti et al., 1993b). These amino acid changes are caused by point mutations or deletions at 15 nucleotides or 8 amino acid codons respectively scattered over a stretch of 67 nucleotides or 23 amino acid codons.

Telenti et al. (1993a and b) described a PCR-SSCP method to screen for the relevant mutations responsible for rifampicin resistance (SSCP refers to single-strand conformation polymorphism). SSCP analysis can be performed either by using radio-activity or by using fluorescent markers. In the latter case sophisticated and expensive equipment (an automated DNA-sequencing apparatus) is needed. The SSCP approach described has also other limitations with respect to specificity and sensitivity which might impede its routine use. Specimen can only be adequatly analysed directly if a significant load of bacteria (microscopy score : >90 organisms/field) is observed microscopically and on crude DNA samples strand-separation artefacts may be observed which complicate the interpretation of the results.

Kapur et al. (1994) describe 23 distinct rpoB alleles associated with rifampicin resistance. In addition to the mutations described by Telenti et al. (1993a), some new mutant rpoB alleles are described. However, the most frequently occuring alleles remain the same as those described before.

In M. leprae, the molecular basis for rifampicin resistance was described by Honoré and Cole (1993). Here too, resistance stemmed from mutations in the rpoB gene, which encodes the beta subunit of RNA polymerase of M. leprae. Only a limited number of resistant M. leprae strains (9) were analysed, and in most of them (8/9) resistance was due to a mutation affecting the Ser-425 residue.

Clinically important mycobacteria other than M. tuberculosis and M. leprae often show an innate, be it variable, resistance to rifampicin. This is the case for M. avium and M. intracellulare, human pathogens for which only limited treatment options are available. Guerrero et al. (1994) compared the rpoB-gene sequences of different M. avium and M. intracellulare isolates with that of M. tuberculosis. Differences are present at the nucleotide level but a full amino acid identity was found with rifampicin-sensitive M. tuberculosis. These findings suggest that another mechanism of resistance, possibly a permeability barrier, applies for M. avium and M. intracellulare.

The specific detection of point mutations or small deletions can elegantly be

approached using hybridization procedures such as the reverse hybridization assay. However, the complexity observed in the relevant part of the rpoB gene does not allow a straightforward probe development. As will be exemplified further. it was one of the objects of the present invention to design a specific approach allowing the detection of most if not all mutations found so far in a fast and convenient way without the need for sophisticated equipment.

The mechanism of resistance to isoniazid (INH) is considerably more complex than that for rifampicin. At least two gene products are involved in INH-resistance. First, there is catalase-peroxidase which is believed to convert INH to an activated molecule. Hence, strains which do not produce catalase-peroxidase by virtue of a defective or deleted katG gene are not anymore susceptible to INH (Zhang et al.. 1992: Stoeckle et al.. 1993). In this context it should be mentioned that the association between INH-resistance and the loss of catalase activity was already noted in the fifties (Middlebrook. 1954 a and b; Youatt. 1969).

The second molecule involved is the inhA gene product. which is believed to play a role in the mycolic acid biosynthesis. It is postulated that the activated INH molecule interacts either directly or indirectly with this product and probably prevents proper mycolic acid biosynthesis. This hypothesis is based on the recent observation that overexpression of the wild type inhA gene or a particular amino acid change (S94A) in the inhA gene product confers resistance to INH (Banerjee et al., 1994).

In short. and somewhat simplified we can state that in certain M. tuberculosis strains resistance to INH might be mediated by :

- the loss of catalase-peroxidase activity
- the presence of certain amino acid changes in the inhA protein
- the expression level of the wild type inhA protein

Also. other mechanisms might be involved in confering resistance to INH and related drugs. The importance of these factors in the total spectrum of INH-resistance mechanisms has yet to be assessed. This issue can be addressed by means of DNA probe techniques if reliable DNA probes can be developed from the available DNA-sequences of the katG gene (EMBL n° X68081) and inhA gene (EMBL n° U02492) of M. tuberculosis. These probe-tests could then also be applied for detection of drug resistance in biological samples.

For the detection of resistance to streptomycine and (fluoro)quinolones the same approach as for rifampicin can be followed. Resistance to these antibiotics is also induced

by point mutations in a limited region of one or more genes. Point mutations in the gyrase gene confer resistance to (fluoro)quinolones (EMBL n° L27512). Streptomycin resistance is correlated with mutations in either the 16S rRNA gene or the gene of a ribosomal protein S12 (rpsL) (Finken et al., 1993: Douglas and Steyn. 1993: Nair et al.. 1993).

Resistance due to nucleotide changes in the katG, rpoB and rpsL genes have been described in international application WO 93/22454. For each of the different genes in M. tuberculosis only one of the many possible mutations was specified in detail. nl. R461L for katG. S425L (equivalent to S531L described by Telenti et al. and the present invention) for rpoB and K42R for rpsL.

It is an object of the present invention to provide a rapid and reliable detection approach for determination of antibiotic resistance of mycobacterial species present in a biological sample.

More particularly, it is an aim of the present invention to provide a rapid and reliable method for determination of resistance to rifampicin (and/or rifabutin) of M. tuberculosis present in a biological sample.

It is also an object of the present invention to provide methods enabling the detection and identification of Mycobacterium species in a biological sample. directly coupled to the monitoring of the antibiotic resistance spectrum.

It is more particularely an aim of the present invention to provide a method to detect the presence of Mycobacterium tuberculosis in a biological sample directly coupled to the detection of rifampicin (and/or rifabutin) resistance.

It is more particularely an aim of the present invention to provide a method to detect the presence of Mycobacterium leprae in a biological sample directly coupled to the detection of rifampicin (and/or rifabutin) resistance.

It is also an aim of the present invention to select particular probes able to discriminate wild-type sequences from mutated sequences conferring resistance to one or more drugs.

It is more particularly an aim of the present invention to select particular probes able to discriminate wild-type sequences from mutated sequences conferring resistance to rifampicin (and/or rifabutin).

It is more particularly an aim of the present invention to select a particular set of probes. able to discriminate wild-type sequences from mutated sequences conferring

resistance to rifampicin (an/or rifabutin) with this particular set of probes being used under the same hybridisation and wash-conditions.

It is moreover an aim of the present invention to combine a set of selected probes able to discriminate wild-type sequences from mutated sequences conferring resistance to rifampicin (and/or rifabutin) with another set of selected probes able to identify the mycobacteria species present in the biological sample. whereby all probes can be used under the same hybridisation and wash-conditions.

It is also an aim of the present invention to select primers enabling the amplification of the gene fragment(s) determining the antibiotic resistance trait of interest.

It is more particularly an aim of the present invention to select primers enabling the amplification of the rpoB-gene fragment determining resistance to rifampicin (and analogues).

Another aim of the invention is to provide kits for the detection of antibiotic resistance in mycobacteria. possibly coupled to the identification of the mycobacterial species involved.

All the aims of the present invention have been met by the following specific embodiments.

The selection of the preferred probes of the present invention is based on the Line Probe Assay (LiPA) principle which is a reverse hybridization assay using oligonucleotide probes immobilized as parallel lines on a solid support strip (Stuyver et al. 1993; international application WO 94/12670). This approach is particularly advantageous since it is fast and simple to perform. The reverse hybridization format and more particularly the LiPA approach has many practical advantages as compared to other DNA techniques or hybridization formats. especially when the use of a combination of probes is preferable or unavoidable to obtain the relevant information sought.

It is to be understood. however, that any other type of hybridization assay or format using any of the selected probes as described further in the invention. is also covered by the present invention.

The reverse hybridization approach implies that the probes are immobilized to a solid support and that the target DNA is labelled in order to enable the detection of the hybrids formed.

The following definitions serve to illustrate the terms and expressions used in the present invention.

The target material in these samples may either be DNA or RNA e.g. genomic DNA

or messenger RNA or amplified versions thereof. These molecules are also termed polynucleic acids.

The term "probe" refers to single stranded sequence-specific oligonucleotides which have a sequence which is complementary to the target sequence to be detected.

The term complementary as used herein means that the sequence of the single stranded probe is exactly complementary to the sequence of the single-stranded target, with the target being defined as the sequence where the mutation to be detected is located. Since the current application requires the detection of single basepair mismatches, very stringent conditions for hybridization are required, allowing in principle only hybridization of exactly complementary sequences. However, variations are possible in the length of the probes (see below), and it should be noted that, since the central part of the probe is essential for its hybridization characteristics, possible deviations of the probe sequence versus the target sequence may be allowable towards head and tail of the probe, when longer probe sequences are used. These variations, which may be conceived from the common knowledge in the art, should however always be evaluated experimentally, in order to check if they result in equivalent hybridization characteristics than the exactly complementary probes.

Preferably, the probes are about 5 to 50 nucleotides long, more preferably from about 10 to 25 nucleotides. The nucleotides as used in the present invention may be ribonucleotides, deoxyribonucleotides and modified nucleotides such as inosine or nucleotides containing modified groups which do not essentially alter their hybridisation characteristics. Probe sequences are represented throughout the specification as single stranded DNA oligonucleotides from the 5' to the 3' end. It is obvious to the man skilled in the art that any of the below-specified probes can be used as such, or in their complementary form, or in their RNA form (wherein T is replaced by U).

The probes according to the invention can be prepared by cloning of recombinant plasmids containing inserts including the corresponding nucleotide sequences, if need be by cleaving the latter out from the cloned plasmids upon using the adequate nucleases and recovering them, e.g. by fractionation according to molecular weight. The probes according to the present invention can also be synthesized chemically, for instance by the conventional phospho-triester method.

The term "solid support" can refer to any substrate to which an oligonucleotide probe can be coupled, provided that it retains its hybridization characteristics and provided that the

background level of hybridization remains low. Usually the solid substrate will be a microtiter plate, a membrane (e.g. nylon or nitrocellulose) or a microsphere (bead). Prior to application to the membrane or fixation it may be convenient to modify the nucleic acid probe in order to facilitate fixation or improve the hybridization efficiency. Such modifications may encompass homopolymer tailing, coupling with different reactive groups such as aliphatic groups, NH_2 groups, SH groups, carboxylic groups, or coupling with biotin, haptens or proteins.

The term "labelled" refers to the use of labelled nucleic acids. Labelling may be carried out by the use of labelled nucleotides incorporated during the polymerase step of the amplification such as illustrated by Saiki et al. (1988) or Bej et al. (1990) or labelled primers, or by any other method known to the person skilled in the art. The nature of the label may be isotopic (^{32}P, ^{35}S, etc.) or non-isotopic (biotin, digoxigenin, etc.).

The term "primer" refers to a single stranded oligonucleotide sequence capable of acting as a point of initiation for synthesis of a primer extension product which is complementary to the nucleic acid strand to be copied. The length and the sequence of the primer must be such that they allow to prime the synthesis of the extension products. Preferably the primer is about 5-50 nucleotides long. Specific length and sequence will depend on the complexity of the required DNA or RNA targets, as well as on the conditions of primer use such as temperature and ionic strenght.

The fact that amplification primers do not have to match exactly with the corresponding template sequence to warrant proper amplification is amply documented in the literature (Kwok et al., 1990).

The amplification method used can be either polymerase chain reaction (PCR; Saiki et al., 1988), ligase chain reaction (LCR: Landgren et al., 1988: Wu & Wallace, 1989; Barany, 1991), nucleic acid sequence-based amplification (NASBA; Guatelli et al., 1990; Compton, 1991), transcription-based amplification system (TAS: Kwoh et al., 1989), strand displacement amplification (SDA; Duck, 1990; Walker et al., 1992) or amplification by means of Qß replicase (Lizardi et al., 1988; Lomeli et al., 1989) or any other suitable method to amplify nucleic acid molecules known in the art.

The oligonucleotides used as primers or probes may also comprise nucleotide analogues such as phosphorothiates (Matsukura et al., 1987), alkylphosphorothiates (Miller et al., 1979) or peptide nucleic acids (Nielsen et al., 1991; Nielsen et al., 1993) or may

contain intercalating agents (Asseline et al.. 1984).

As most other variations or modifications introduced into the original DNA sequences of the invention these variations will necessitate adaptions with respect to the conditions under which the oligonucleotide should be used to obtain the required specificity and sensitivity. However the eventual results of hybridisation will be essentially the same as those obtained with the unmodified oligonucleotides.

The introduction of these modifications may be advantageous in order to positively influence characteristics such as hybridization kinetics. reversibility of the hybrid-formation, biological stability of the oligonucleotide molecules. etc.

The "sample" may be any biological material taken either directly from the infected human being (or animal), or after culturing (enrichment). Biological material may be e.g. expectorations of any kind. broncheolavages, blood. skin tissue. biopsies. lymphocyte blood culture material. colonies. liquid cultures. soil. faecal samples. urine etc.

The probes of the invention are designed for attaining optimal performance under the same hybridization conditions so that they can be used in sets for simultaneous hybridization; this highly increases the usefulness of these probes and results in a significant gain in time and labour. Evidently, when other hybridization conditions would be preferred. all probes should be adapted accordingly by adding or deleting a number of nucleotides at their extremities. It should be understood that these concommitant adaptations should give rise to essentially the same result. namely that the respective probes still hybridize specifically with the defined target. Such adaptations might also be necessary if the amplified material should be RNA in nature and not DNA as in the case for the NASBA system.

For designing probes with desired characteristics. the following useful guidelines known to the person skilled in the art can be applied.

Because the extent and specificity of hybridization reactions such as those described herein are affected by a number of factors. manipulation of one or more of those factors will determine the exact sensitivity and specificity of a particular probe. whether perfectly complementary to its target or not. The importance and effect of various assay conditions. explained further herein. are known to those skilled in the art.

First. the stability of the [probe : target] nucleic acid hybrid should be chosen to be compatible with the assay conditions. This may be accomplished by avoiding long AT-rich sequences. by terminating the hybrids with G:C base pairs. and by designing the probe with

an appropriate Tm. The beginning and end points of the probe should be chosen so that the length and %GC result in a Tm about 2-10°C higher than the temperature at which the final assay will be performed. The base composition of the probe is significant because G-C base pairs exhibit greater thermal stability as compared to A-T base pairs due to additional hydrogen bonding. Thus, hybridization involving complementary nucleic acids of higher G-C content will be stable at higher temperatures.

Conditions such as ionic strenght and incubation temperature under which a probe will be used should also be taken into account when designing a probe. It is known that hybridization will increase as the ionic strenght of the reaction mixture increases, and that the thermal stability of the hybrids will increase with increasing ionic strenght. On the other hand, chemical reagents, such as formamide, urea, DMSO and alcohols, which disrupt hydrogen bonds, will increase the stringency of hybridization. Destabilization of the hydrogen bonds by such reagents can greatly reduce the Tm. In general, optimal hybridization for synthetic oligonucleotide probes of about 10-50 bases in length occurs approximately 5°C below the melting temperature for a given duplex. Incubation at temperatures below the optimum may allow mismatched base sequences to hybridize and can therefore result in reduced specificity.

It is desirable to have probes which hybridize only under conditions of high stringency. Under high stringency conditions only highly complementary nucleic acid hybrids will form; hybrids without a sufficient degree of complementarity will not form. Accordingly, the stringency of the assay conditions determines the amount of complementarity needed between two nucleic acid strands forming a hybrid. The degree of stringency is chosen such as to maximize the difference in stability between the hybrid formed with the target and the nontarget nucleic acid. In the present case, single base pair changes need to be detected, which requires conditions of very high stringency.

Second, probes should be positioned so as to minimize the stability of the [probe : nontarget] nucleic acid hybrid. This may be accomplished by minimizing the length of perfect complementarity to non-target organisms, by avoiding GC-rich regions of homology to non-target sequences, and by positioning the probe to span as many destabilizing mismatches as possible. Whether a probe sequence is useful to detect only a specific type of organism depends largely on the thermal stability difference between [probe:target] hybrids and [probe:nontarget] hybrids. In designing probes, the differences in these Tm values should be

as large as possible (e.g. at least 2°C and preferably 5°C).

The length of the target nucleic acid sequence and, accordingly, the length of the probe sequence can also be important. In some cases, there may be several sequences from a particular region, varying in location and length, which will yield probes with the desired hybridization characteristics. In other cases, one sequence may be significantly better than another which differs merely by a single base. While it is possible for nucleic acids that are not perfectly complementary to hybridize, the longest stretch of perfectly complementary base sequence will normally primarily determine hybrid stability. While oligonucleotide probes of different lengths and base composition may be used, preferred oligonucleotide probes of this invention are between about 5 to 50 (more particularely 10-25) bases in length and have a sufficient stretch in the sequence which is perfectly complementary to the target nucleic acid sequence.

Third, regions in the target DNA or RNA which are known to form strong internal structures inhibitory to hybridization are less preferred. Likewise, probes with extensive self-complementarity should be avoided. As explained above, hybridization is the association of two single strands of complementary nucleic acids to form a hydrogen bonded double strand. It is implicit that if one of the two strands is wholly or partially involved in a hybrid that it will be less able to participate in formation of a new hybrid. There can be intramolecular and intermolecular hybrids formed within the molecules of one type of probe if there is sufficient self complementarity. Such structures can be avoided through careful probe design. By designing a probe so that a substantial portion of the sequence of interest is single stranded, the rate and extent of hybridization may be greatly increased. Computer programs are available to search for this type of interaction. However, in certain instances, it may not be possible to avoid this type of interaction.

The present invention provides in its most general form for a method to detect the antibiotic resistance spectrum of Mycobacterium species present in a sample, possibly coupled to the identification of the Mycobacterium species involved, comprising the steps of:

(i) if need be releasing, isolating or concentrating the polynucleic acids present in the sample:

(ii) if need be amplifying the relevant part of the antibiotic resistance genes present in said sample with at least one suitable primer pair;

(iii) hybridizing the polynucleic acids of step (i) or (ii) with at least one of the rpoB gene

probes as mentioned in table 2, under appropiate hybridization and wash conditions:

(iv) detecting the hybrids formed in step (iii):

(v) inferring the Mycobacterium antibiotic resistance spectrum. and possibly the Mycobacterium species involved from the differential hybridization signal(s) obtained in step (iv).

The relevant part of the antibiotic resistance genes refers to the regions in the rpoB, katG, inhA, 16S rRNA, rpsL and gyrase genes harboring mutations causing resistance to rifampicin, isoniazid, streptomycin and (fluoro)quinolones as described above.

According to a preferred embodiment of the present invention. step (iii) is performed using a set of probes meticulously designed as such that they show the desired hybridization results. when used under the same hybridization and wash conditions.

More specifically. the present invention provides a method for detection of rifampicin (and/or rifabutin) resistance of M. tuberculosis present in a biological sample. comprising the steps of :

(i) if need be releasing, isolating or concentrating the polynucleic acids present in the sample;

(ii) if need be amplifying the relevant part of the rpoB gene present in said polynucleic acids with at least one suitable primer pair:

(iii) hybridizing the polynucleic acids of step (i) or (ii) with a selected set of rpoB wild-type probes under appropiate hybridization and wash conditions. with said set comprising at least one of the following probes (see Table 2) :

S1	(SEQ ID N0 1)
S11	(SEQ ID N0 2)
S2	(SEQ ID N0 3)
S3	(SEQ ID N0 4)
S33	(SEQ ID N0 5)
S4	(SEQ ID N0 6)
S44	(SEQ ID N0 7)
S444	(SEQ ID N0 43)
S4444	(SEQ ID N0 8)
S5	(SEQ ID N0 9)
S55	(SEQ ID N0 10)

S555	(SEQ ID N0 39)
S5555	(SEQ ID N0 40)
S55C	(SEQ ID N0 44)
S55M	(SEQ ID N0 45)
S6	(SEQ ID N0 11)
S66	(SEQ ID N0 12)

(iv) detecting the hybrids formed in step (iii):

(v) inferring the rifampicin susceptibility (sensitivity versus resistance) of M. tuberculosis present in the sample from the differential hybridization signal(s) obtained in step (iv).

The term 'susceptibility' refers to the phenotypic characteristic of the M. tuberculosis strain being either resistant or sensitive to the drug, as determined by *in vitro* culture methods, more specifically to rifampicin (and/or rifabutin). Resistance to rifampicin is revealed by the absence of hybridization with at least one of the S-probes.

Standard hybridization and wash conditions are for instance 3X SSC (Sodium Saline Citrate), 20% deionized FA (Formamide) at 50°C. Other solutions (SSPE (Sodium saline phosphate EDTA), TMACI (Tetramethyl ammonium Chloride), etc.) and temperatures can also be used provided that the specificity and sensitivity of the probes is maintained. If need be, slight modifications of the probes in length or in sequence have to be carried out to maintain the specificity and sensitivity required under the given circumstances. Using the probes of the invention, changing the conditions to 1.4X SSC, 0.07% SDS at 62°C lead to the same hybridisation results as those obtained under standard conditions, without the necessity to adapt the sequence or length of the probes.

Suitable primer pairs can be chosen from a list of primer pairs as described below.

In a more preferential embodiment, the above-mentioned polynucleic acids from step (i) or (ii) are hybridized with at least two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen or seventeen of the above-mentioned S-probes, preferably with 5 or 6 S-probes, which, taken together, cover the "mutation region" of the rpoB gene.

The term "mutation region" means the region in the rpoB-gene sequence where most, if not all of the mutations responsible for rifampicin resistance are located. This mutation region is represented in fig. 1.

In a more preferential embodiment, the above-mentioned polynucleic acids from step (i) or (ii) are hybridized with a selected set of rpoB-wild-type probes, with said set comprising at least one, and preferentially all of the following probes (see Table 2) :

S11 (SEQ ID N0 2)
S2 (SEQ ID N0 3)
S33 (SEQ ID N0 5)
S4444 (SEQ ID N0 8)
S55 or S5555 (SEQ ID N0 10 or 40)

In another particular embodiment the set of S-probes as described above, or at least one of them, can be combined with one or more SIL-probes, detecting silent mutations in the rpoB gene. A preferential SIL-probe is SIL-1 (SEQ ID N0 13, see Table 2).

In another embodiment of the invention, the set of S-probes and possibly SIL-probes, can be combined with at least one R-probe detecting a specific mutation associated with rifampicin-resistance.

R-probes are selected from the following group of probes (see Table 2) :

R1 (SEQ ID N0 46)
R2 (SEQ ID N0 14)
R2B (SEQ ID N0 47)
R2C (SEQ ID N0 48)
R3 (SEQ ID N0 49)
R4A (SEQ ID N0 15)
R44A (SEQ ID N0 16)
R444A (SEQ ID N0 17)
R4B (SEQ ID N0 18)
R44B (SEQ ID N0 19)
R444B (SEQ ID N0 20)
R4C (SEQ ID N0 50)
R4D (SEQ ID N0 51)
R4E (SEQ ID N0 52)
R5 (SEQ ID N0 21)
R55 (SEQ ID N0 22)
R5B (SEQ ID N0 53)

R5C (SEQ ID N0 54)

Preferably the set of S-probes and possibly SIL-probes can be combined with at least two. three, four, five. six. or more R probes.

Most preferably, the set of S-probes and possibly SIL-probes are combined with at least one R-probe from the following restricted group of probes :

R2 (SEQ ID N0 14)

R444A (SEQ ID N0 17)

R444B (SEQ ID N0 20)

R55 (SEQ ID N0 22)

In the case where S and R probes are combined. resistance to rifampicin is revealed by absence of hybridization with one of the S-probes and possibly by a positive hybridization signal with the corresponding R-probe.

Since some mutations may be more frequently occurring than others. e.g. in certain geographic areas (see e.g. Table 5) or in specific circumstances (e.g. rather closed communities) it may be appropiate to screen only for specific mutations. using a selected set of S and/or R probes. This would result in a more simple test. which would cover the needs under certain circumstances. According to Telenti et al. (1993a and b) most mutations described in his publication are relatively rare (3% or less): predominant mutations are S531L (51.6%). H526Y (12.5%). D516V (9.4%) and H526D (7.8%).

In a particular embodiment of the invention a selected set of two or three S-probes is used. the respective sets of probes being:

S4444 (SEQ ID N0 8)

S55 (or S5555) (SEQ ID N0 10 or 40)

or

S2 (SEQ ID N0 3)

S4444 (SEQ ID N0 8)

S55 (or S5555) (SEQ ID N0 10 or 40)

Using this restricted sets of S-probes the majority of rifampicin resistant cases can be detected. by an absence of hybridisation signal with at least one of these probes.

In another particular embodiment of the invention. at least one R probe is used. possibly combined with a selected set of two or three S probes as described above. said R probe being chosen from the following list of probes:

R2 (SEQ ID N0 14)
R444A (SEQ ID N0 17)
R444B (SEQ ID N0 20)
R55 (SEQ ID N0 22)

In this case, the specific mutation responsible for the rifampicin-resistant phenotype can be inferred from a positive hybridization signal with one of the R-probes and/or the absence of hybridization with the corresponding S-probe.

In another embodiment of the invention, the above-mentioned S, SIL or R probes may be combined with at least one species-specific probe for M. tuberculosis allowing simultaneous identification of Mycobacterium tuberculosis and detection of rifampicin resistance, with said species-specific probe being chosen from the following group of probes (see Table 2) :

MT-POL-1 (SEQ ID N0 23)
MT-POL-2 (SEQ ID N0 24)
MT-POL-3 (SEQ ID N0 25)
MT-POL-4 (SEQ ID N0 26)
MT-POL-5 (SEQ ID N0 27)

Most preferably the species-specific M. tuberculosis probe is:

MT-POL-1 (SEQ ID N0 23)

In yet another embodiment of the invention, the above-mentioned S, SIL, R or MT-POL probes can be combined with at least one species-specific probe for M. paratuberculosis, M. avium, M. scrophulaceum, M. kansasii, M. intracellulare (and MAC-strains) or M. leprae, with said probes being respectively MP-POL-1 (SEQ ID N0 28), MA-POL-1 (SEQ ID N0 29), MS-POL-1 (SEQ ID N0 38), MK-POL-1 (SEQ ID N0 55), MI-POL-1 (SEQ ID N0 68) and ML-POL-1 (SEQ ID N0 57) (see table 2B) or any species-specific probe derived from the sequence of the relevant part of the rpoB gene of M. paratuberculosis (SEQ ID N0 35), M. avium (SEQ ID N0 36), M. scrofulaceum (SEQ ID N0 37), M. kansasii (SEQ ID N0 56) or MAC-strains (SEQ ID N0 69), as represented respectively in figs. 5, 6, 7, 8 and 11. It should be noted that the sequences represented in figs. 5-8 and 11 are new. The sequence of the rpoB-gene fragment of M. intracellulare and M. leprae has been described by others (Guerrero et al. 1994: Honore and Cole. 1993).

The term "MAC-strains" means "M. avium complex" strains known to the man

skilled in the art of mycobacteria taxonomy. This rather heterogeneous group of MAC-strains may however comprise strains which are genotypically rather like M. intracellulare. This is also the case for isolate ITG 926, of which the rpoB sequence is shown in fig. 11. The MI-POL-1 probe derived from SEQ ID N0 69 and the published M. intracellulare rpoB sequence is therefor specific for M. intracellulare and some MAC strains together.

It should be stressed that all of the above-mentioned probes. including the species-specific probes, are contained in the sequence of the rpoB gene. and more particulary in the sequence of the amplified rpoB gene fragment. Moreover. as illustrated further in the examples. the probes described above as "preferential". are designed in such a way that they can all be used simultaneously, under the same hybridization and wash conditions. These two criteria imply that a single amplification and hybridization step is sufficient for the simultaneous detection of rifampicin resistance and the identification of the mycobacterial species involved.

In a preferential embodiment, and by way of an example. a method is disclosed to detect M. tuberculosis and its resistance to rifampicin. comprising the steps of:

(i) if need be releasing, isolating or concentrating the polynucleic acids present in the sample;

(ii) if need be amplifying the relevant part of the rpoB gene with at least one suitable primer pair;

(iii) hybridizing the polynucleic acids of step (i) or (ii) with the following set of probes under appropriate hybridization and wash conditions

MT-POL-1

S11

S2

S33

S4444

S55 or S5555

R2

R444A

R444B

R55

(iv) detecting the hybrids formed in step (iii):

(v) inferring the presence of M. tuberculosis and its susceptibility to rifampicin from the differential hybridization signal(s) obtained in step (iv).

In order to detect the mycobacterial organisms and/or their resistance pattern with the selected set of oligonucleotide probes, any hybridization method known in the art can be used (conventional dot-blot, Southern blot, sandwich, etc.).

However, in order to obtain fast and easy results if a multitude of probes are involved, a reverse hybridization format may be most convenient.

In a preferred embodiment the selected set of probes are immobilized to a solid support. In another preferred embodiment the selected set of probes are immobilized to a membrane strip in a line fashion. Said probes may be immobilized individually or as mixtures to delineated locations on the solid support.

A specific and very user-friendly embodiment of the above-mentioned preferential method is the LiPA method, where the above-mentioned set of probes is immobilized in parallel lines on a membrane, as further described in the examples.

The above mentioned R-probes detect mutations which have already been described in the prior art (Telenti et al., 1993a and 1993b). However, as illustrated further in the examples, four new mutations associated with rifampicin-resistance in M. tuberculosis, not yet described by others, are disclosed by the current invention. Using the S-probes of the current invention, new mutations as well as mutations already described in the prior art can be detected. The unique concept of using a set of S-probes covering the complete mutation region in the rpoB gene, allows to detect most, if not all of the mutations in the rpoB-gene responsible for rifampicin resistance, even those mutations which would not yet have been described uptil now.

The four new rpoB mutations (D516G, H526C, H526T and R529Q) are marked in Table 1 with an asterisk. By way of an example, the sequence of the rpoB-mutant allele H526C is represented in Fig. 2 (SEQ ID N0 34).

The invention also provides for any probes or primersets designed to specifically detect or amplify specifically these new rpoB gene mutations, and any method or kits using said primersets or probes.

In another embodiment, the invention also provides for a method for detection of rifampicin (and/or rifabutin) resistance of M. leprae present in a biological sample, comprising the steps of:

(i) if need be releasing. isolating or concentrating the polynucleic acids present in the sample;

(ii) if need be amplifying the relevant part of the rpoB gene with at least one suitable primer pair:

(iii) hybridizing the polynucleic acids of step (i) or (ii) with a selected set of rpoB wild-type probes under appropiate hybridization and wash conditions. with said set comprising at least one of the following probes (see Table 2) :

ML-S1 (SEQ ID N0 58)
ML-S2 (SEQ ID N0 59)
ML-S3 (SEQ ID N0 60)
ML-S4 (SEQ ID N0 61)
ML-S5 (SEQ ID N0 62)
ML-S6 (SEQ ID N0 63)

(iv) detecting the hybrids formed in step (iii):

(v) inferring the rifampicin susceptibility (sensitivity versus resistance) of M. leprae present in the sample from the differential hybridization signal(s) obtained in step (iv).

Resistance to rifampicin is revealed by the absence of hybridization with at least one of the ML-S-probes.

In another embodiment of the invention. the above-mentioned ML-S probes may be combined with a species specific probe for M. leprae. ML-POL-1. allowing simultaneous identification of M. leprae and detection of rifampicin resistance. with said species specific probe being represented in SEQ ID N0 57.

It is to be noted that the above-mentioned ML-S probes and ML-POL-1 probe are all contained within the same amplified rpoB-gene fragment of M. leprae. and are designed as such that they can all be used under the same hybridization and wash conditions.

The invention further provides for any of the probes as described above. as wemm as compositions comprising at least one of these probes.

The invention also provides for a set of primers. allowing amplification of the mutation region of the rpoB gene of M. tuberculosis. The sets of primers can be chosen from the following group of sets (see table 2):

P1 and P5 (SEQ ID N0 30 and 33)

P3 and P4 (SEQ ID N0 31 and 32)

P7 and P8 (SEQ ID N0 41 and 42)

P2 and P6, in combination with (P1 and P5) or (P3 and P4) or (P7 and P8)

Most preferably, the set of primers is the following:

P3 and P4 (SEQ ID N0 31 and 32).

The invention also provides for a set of primers allowing amplification of the mutation region of the rpoB gene in mycobacteria in general, i.e. in at least M. tuberculosis, M. avium, M. paratuberculosis, M. intracellulare, M. leprae, M. scrofulaceum. These general primers can be used e.g. in samples where the presence of mycobacteria other than M. tuberculosis is suspected, and where it is desirable to have a more general detection method. The set of primers is composed of a 5`-primer, selected from the following set:

MGRPO-1 (SEQ ID N0 64)

MGRPO-2 (SEQ ID N0 65)

and a 3`-primer, selected from the following set:

MGRPO-3 (SEQ ID N0 66)

MGRPO-4 (SEQ ID N0 67).

The sequence of these primers is shown in Table 2B.

Primers may be labeled with a label of choice (e.g. biotine). Different primer-based target amplification systems may be used, and preferably PCR-amplification, as set out in the examples. Single-round or nested PCR may be used.

The invention also provides for a kit for inferring the antibiotic resistance spectrum of mycobacteria present in a biological sample, possibly coupled to the identification of the mycobacterial species involved, comprising the following components:

(i) when appropiate, a means for releasing, isolating or concentrating the polynucleic acids present in the sample;

(ii) when appropriate, at least one of the above-defined set of primers;

(iii) at least one of the probes as defined above, possibly fixed to a solid support;

(iv) a hybridization buffer, or components necessary for producing said buffer;

(v) a wash solution, or components necessary for producing said solution;

(vi) when appropriate, a means for detecting the hybrids resulting from the preceding hybridization.

The term "hybridization buffer" means a buffer enabling a hybridization reaction to

occur between the probes and the polynucleic acids present in the sample, or the amplified products, under the appropiate stringency conditions.

The term "wash solution" means a solution enabling washing of the hybrids formed under the appropiate stringency conditions.

More specifically, the invention provides for a kit as described above, for the simultaneous detection of M. tuberculosis and its resistance to rifampicin.

In another specific case, the invention provides for a kit as described above, for the simultaneous detection of M. leprae and its resistance to rifampicin.

TABLE LEGENDS

Table 1 summarizes the nucleotide and amino acid changes (described by Telenti et al. (1993a and b), Kapur et al. 1994 and the present invention) in the rpoB gene fragment of rifampicin resistant M. tuberculosis isolates. Codon numbering is as in Fig. 1. New mutations described by the current invention are indicated with an asterisk (*).

Table 2 lists the sequences of the primers and probes selected from the rpoB gene.

2A: M. tuberculosis

2B: other mycobacterial species

Table 3 shows the hybridization results obtained with probe MT-POL-1. with DNA from different mycobacterial and non-mycobacterial species.

Table 4 shows some representative results obtained with LiPA for some M. tuberculosis isolates which have been sequenced and the interpretation of the different LiPA patterns.

Table 5 shows the occurrence of the different rpoB mutations in M. tuberculosis in different geographical areas. Abbreviations: Bel= Belgium. Bengla= Bengladesh. Bur-Fa= Burkina faso. Buru= Burundi, Can= Canada. Chi= Chili. Col= Colombia. Egy= Egypt, Gui= Guinea. Hon= Honduras. Pak= Pakistan. Rwa= Rwanda. Tun= Tunesia.

Table 6 shows a comparison of LiPA results versus rifampicin resistance determination in culture for M. tuberculosis. S= Sensitive. R= Resistant.

FIGURE LEGENDS

Fig. 1 represents the nucleotide sequence and the amino acid sequence of the mutation region of respectively the rpoB gene and the RNA polymerase ß-subunit of a wild-type (i.e. not resistant) Mycobacterium tuberculosis strain (ITG 9081). The codon (amino acid) numbering is as in Telenti et al. (1993a). The nucleotides or amino acids involved in resistance-inducing changes are underlined. The observed mutations are boxed. The horizontal bars indicate the positions of some of the oligonucleotide probes (one probe per group is indicated).

Fig 2 shows the partial nucleotide sequence of the newly described rpoB mutant allele of M. tuberculosis strain ITG 9003 (SEQ ID N0 34).

Fig 3 shows results obtained on LiPA-strips with probes S44 and S4444 applied at different concentrations on the membrane strip. As target material, nucleic acid preparations of M. tuberculosis strains ITG 8872 and ITG 9081 were used.

Fig 4 gives a comparison of the performance of probes S44 and S4444 in a Line probe assay conformation. The probes on strip A and B are identical except for S44 and S4444. The hybridizations were performed using amplified material originating from M. tuberculosis strain ITG 8872.

Fig 5 shows the partial nucleotide sequence of the presumptive rpoB gene of M. paratuberculosis strain 316F (SEQ ID N0 35)

Fig 6 shows the partial nucleotide sequence of the presumptive rpoB gene of M. avium strain ITG 5887 (SEQ ID N0 36)

Fig 7 shows the partial nucleotide sequence of the presumptive rpoB gene of M. scrofulaceum strain ITG 4979 (SEQ ID N0 37)

Fig 8 shows the partial nucleotide sequence of the presumptive rpoB gene of M. kansasii strain ITG 4987 (SEQ ID N0 56)

Fig 9 shows some rpoB mutations in M. tuberculosis and their corresponding LiPA patterns. Nomenclature of the mutations is as described in Table 1. Nomenclature of the LiPA pattern is as follows:

wt= positive hybridization with all S-probes, and negative hybridization with all R-probes:

ΔS1-5= absence of hybridization with the respective S-probe:

R2, 4A, 4B, 5= positive hybridization with the respective R-probes and absence of